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(57) Abstract

A population of beads having a density > 1 g/cm^{-3} and comprising a polymer base matrix in which a particulate filler is incorporated. The beads are characterized in that the filler particles have a density $\geq$ 3 g/cm^{-3} and in that the density and/or size of the beads are distributed within predetermined density and size ranges. Particularly important filler particles are those which have rounded shapes, for instance spheres, ellipsoids or aggregates/agglomerates thereof. The bead population is particularly usable in adsorption processes in fluidized beds, with preference to stable expanded beds.

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ADSORPTION METHOD AND SEPARATION MEDIUM

TECHNICAL FIELD AND EARLIER KNOWN TECHNIQUES

The invention relates to adsorption processes, particularly
5 chromatography, on expanded/fluidized beds. The most advanta-
geous bed is generated by expanding/fluidizing a bed
consisting of sedimented beads by directing a flow of fluid
against the force causing sedimentation. When the bed has
been expanded or fluidized to the volume desired, a sample is
10 introduced into the fluidizing flow. The sample-carried
substance or substances to be separated out are therewith
adsorbed on the beads so as to be delayed in relation to the
sample in its passage through the bed. Adsorption is achieved
by virtue of the ability of the matrix/beads to bind to the
15 substance or substances to be separated out. A less effective
bed can be generated by agitating suspendible beads with the
aid of a turbulent flow or by mechanical agitation. This
latter type of bed does not, of course, refer to chroma-
tography, but to a batch-wise adsorption process.
20   In the context of the present invention the term "bead"
refers to the population of particles making up the the bed.
Beads may be more or less spherical and encompasses thus
also irregular forms, such as granules, crush-like forms etc,
although in connection with the invention spherical or
25 otherwise rounded forms are preferred.
By selecting a bead population which includes beads of
mutually different sizes and/or densities and using this
population in an expanded bed, it is possible to obtain a so-
called classified bed in which the larger and heavier beads
30 lie furthest down in the bed and the smaller and lighter
particles are located further up in the bed. The beads will
adopt a state of equilibrium when fluid distribution in the
bottom of the fluidizing vessel is uniform. Each bead will
move within a highly limited volume of the total bed, which
35 means that back-mixing of beads will be very limited. This is
the characteristic of a stable expanded bed (sometimes called
a stable fluidized bed) and is in contrast to full back-
mixing which is more normal in the case of fluidized beds. It
is important that back-mixing is kept to a minimum, in order

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to achieve an effective chromatographic process. Reference is made to multi-stage adsorption and the occurrence of concentration gradients of adsorbed substance in the longitudinal direction of the bed. Back-mixing in a bed is often measured as axial dispersion, often expressed by "vessel dispersion number" (see Levenspiel, "Chemical Reaction Engineering" 2nd Edition, John Wiley & Sons (1972)). For stable expanded beds, the vessel dispersion number will preferably be $\leq 75 \times 10^{-3}$, more preferably $\leq 20 \times 10^{-3}$. Back-mixing in the bed can be illustrated with the vessels-in-series model, where each vessel, or tank, represents a theoretical plate (see Levenspiel), "Chemical Reaction Engineering" 2nd Edition, John Wiley & Sons (1972)). The larger the number of plates, the more stable and more effective the bed. The number of plates of a bed is related directly to the vessel dispersion number. The number of plates for an expanded bed intended for chromatography is preferably ≥ 5, more preferably ≥ 30. In a fluidized bed with total back-mixing, the number of plates is 1, i.e. complete agitation.

Expansion/fluidization of the bed is normally effected in a column having provided at its ends a net structure which covers the cross-sectional area of the column, or with some other perforated device which will not generate turbulence in the flow. See, for instance, WO-A-9218237 (Pharmacia Biotech AB, Uppsala, Sweden) in this regard.

Subsequent to adsorption, elution can be effected directly from the expanded bed. Alternatively, the bed may be allowed to settle and adsorbed material eluated from the bed with the aid of a fluid flow normally delivered in a direction opposite to that in which the bed is expanded.

Although aqueous liquids are normally used (for instance buffers dissolved in water), this does not exclude the use of other liquids.

In conjunction with expanded beds, mention is often made of the degree of expansion H/H_0, where H is the height of the expanded bed and H_0 is the height of the sedimented bed. For practical reasons, the lowest possible degree of expansion is chosen, although without permitting the beads to pack

together or form agglomerates. The degree of expansion will normally lie in the range of 2-10, preferably 2.4-3.2, with a priority that the bed will, at the same time, be stable and achieve the number of bottoms necessary in context.

5   There are at present at least two commercial suppliers of systems and/or matrices for chromatography on expanded stable beds. Pharmacia Biotech AB (Uppsala, Sweden) market Stream-line® which utilizes beads of cross-linked polysaccharide (agarose) with quartz particles (similar to glass crush with sharp edges) as filler. The beads have a density of about 1.2

10 with sizes lying in the range of 125-315 μm. Streamline® has been used primarily for ion exchange chromatography. See also WO-A-9218237 (Pharmacia Biotech AB) in which there is given a description of suitable column constructions. The other main

15 supplier is Bioprocessing Ltd. (Durham, England) whose porous glass beads (Prosep®) can be used among other things for chromatography on expanded beds (Beyzavi, et al, Genetic Engineering News, March 1, 1994 17)). Still another supplier in the field is Sepracor.

20 Also described in the patent literature are similar systems for chromatography on unstable fluidized beds, i.e. beds with a smaller requirement on low back-mixing and low axial dispersion. See, for instance, U.S. 4,976,865 (Sanchez, et al, CNRS) which teaches the use of segmented columns and

25 allows turbulence of the beads within each segment to a more or a less pronounced extent. In this variant, a concentration gradient is generated by virtue of allowing a series of batch adsorptions to be carried out with more or less heavily pronounced back-mixing in each segment.

30 WO-A-9200799 (KEM-EN-TEK; Upfront Chromatography) teaches the use of a fluidized bed in which it is endeavoured to generate homogenous intermixing and distribution of flow by agitating the inlet flow to create a mixing zone that is followed by a non-turbulent zone. The non-turbulent zone has

35 later been claimed to behave as a stable expanded bed. The direction of flow may be either upward or downward utilizing beads that are heavier or lighter, respectively, than the fluid utilized. This latter publication discloses a large number of fillers and polymeric materials that can be

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combined to produce beads usable for adsorption in fluidized beds.

PROBLEMS RELATED TO EARLIER FLUIDISED BED SYSTEM

5   In the case of adsorption processes on expanded and/or fluidized beds, there is an expressed desire to use beads that have an improved total capacity and/or breakthrough capacity. The desire for improved breakthrough capacity becomes particularly manifest when wishing to use rates of
10   flow above those that can be achieved with current techniques, for instance rates of flow of 1,000 cm/h and preferably still higher, such as flow rates up to 1,500 cm/h. The requirement of stable expanded beds and low degrees of expansion described above also requires access to beads which
15   are able to form sediments more readily. Beads of higher density are also desirable in batch adsorptions in fluidized beds having a high degree of back-mixing (unstable), since beds of this nature will sediment more rapidly at the end of the adsorption process. There is thus a need for bead
20   populations whose beads have a higher density or are larger than is the case in earlier known techniques. At the same time, an increase in flow rate will in most cases result in a decrease in the breakthrough capacity, which in turn counteracts high productivity in the processes concerned. Parallel
25   herewith is the need for beads that have an improved breakthrough capacity.

In Streamline®, there has been used hitherto a filler matrix comprising quartz particles in an agarose matrix. A total capacity of 24 mg/ml gel for IgG can be obtained when
30   coupling Protein A to the beads. The breakthrough capacity for the same gel at a linear flow rate of 300 cm/h (with c/c_0 = 0.01, where c is the IgG concentration in eluate and c_0 is the IgG concentration in applied liquid) is only 1 mg IgG per ml of gel. In the case of Prosep® (Bioprocessing Ltd.), the
35   concentrations are 30 mg and 24 mg IgG per ml of gel, respectively. This figures refer to study on packed beds but the relation between beads with and without filler is transferrable to expanded beds. This illustrates that filler decreases breakthrough capacity. Although porous glass beads

are able to give a high breakthrough capacity in relation to total capacity, the density cannot be increased to above that which glass itself affords since filler particles cannot be inserted in glass.

OBJECTIVES OF THE INVENTION

o A first objective of the invention is to provide more rapid methods for adsorption/desorption processes that involve fluidized beds. Of particular importance in this regard is chromatography on expanded beds.

o A second objective is to provide filler matrices that have improved breakthrough capacity and are particularly adapted for chromatography on a stable expanded bed.

o A third objective is to make possible high yields in chromatography on stable expanded beds.

DISCLOSURE OF THE INVENTION

We have now discovered that elevated flow rates can be applied and satisfactory breakthrough capacities obtained by using so-called filler matrices and by optimizing the filler with regard to form, diameter, density and quantity. Filler material in the form irregular particles with sharp edges lowers the breakthrough capacity more than spherical granules and other more or less rounded shapes. This does not exclude the fact that the filler matrices should also be optimized with respect to other variables generally accepted for chromatography, for instance the bead pore-sizes, pore volume and diameter. For instance, it is known that increasing pore sizes facilitate diffusion and permeation of substances to be adsorbed on the matrix. It is also known that decreasing bead diameters increase the number of pore openings per unit of gel volume, which in turn enhances the possibility of substances permating the beads (enhances the breakthrough capacity). It is also known that optimal values with regard to pore diameter and bead diameter are also related to the substance to be bound/adsorbed to/on the beads.

A main aspect of the invention resides in an adsorption method as defined in the introduction. This aspect of the invention is characterized in that the beads used contain

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filler in the form of granules or particles having a density ≥ 3 g/cm^3, preferably ≥ 4 g/cm^3, and thereabove. Those fillers that have been studied hitherto have all had a density < 20 g/cm^3. This does not exclude the possibility of utilizing
5 other potential fillers with still higher densities, e.g. up to 25 g/cm^3. It is important that the filler particles are inert and undissolvable in those conditions applied to the use of the beads.

   The material in suitable filler particles is often a heavy
10 metal, either in the form of an alloy such as steel (e.g. Anval®; Anval, Torshälla, Sweden) or an oxide (e.g. zirconium oxide) or some other metal salt (e.g. tungsten carbide). The filler may also comprise metal spheres (e.g. tantalum).

   The filler particles may vary in size and the size of said
15 particles will always be much smaller than the size of the beads used. Typical sizes are 1-70 µm, with a preference to a range of 15-50 µm.

   The geometric shape of the filler is highly significant when wishing to retain a high breakthrough capacity in
20 relation to corresponding beads which lack filler. Thus, preferred filler shapes are spheres, ellipsoids, droplets, noodle shapes, bean shapes and other rounded shapes including aggregates/agglomerates and irregular shapes thereof. A particular preference is given to rounded shapes which are
25 continuously rounded.

   The filler content of the beads is determined by the density to be achieved, i.e. the rates of flow that are conceivable for use.

   When a stable expanded bed is used, the beads should
30 preferably vary in size and/or in density so that they are able to more easily position themselves stably, with lighter and/or smaller beads above heavier and/or larger beads. No compartimentalization in the form of column segmentation is necessary. Neither is it necessary to use magnetic filler in
35 combination with external magnetic fields. Thus, there can be used a bead fraction having sizes within a given range where the proportion of beads in the lower part of the range will be larger than the beads in the upper part thereof.

Typical particle size distributions for a bead population used to create a stable expanded bed are normally such that 95% of the beads fall within a range whose width is 0.1 to 10 times the mean bead diameter, preferrably 0.3 to 3 times the

5 mean bead diameter. The exact particle size distribution to be selected will depend on factors, such as flow rate, mean bead diameter, density of beads, density of fluid etc. A too wide particle size distribution will result in elutriation and/or sedimenting of large proportions of beads. A too

10 narrow   particle   size   distribution   will   counteract stabilization of the expanded bed. This implies that the population of beads can only be monodisperse in case the individual beads of a population have density distribution within a given density interval.

15 The ratio between total surface area the beads (outer pus inner surface) and the total bead volume is highly significant to breakthrough capacity. Larger relative contact surface areas (small beads) lead to a higher breakthrough capacity. The total capacity, on the other hand, is only

20 marginally affected. The mean particle size of the beads should generally lie in the range of 10-1,000 µm, with preference to a range of 50-700 µm. The lower limit is determined with a view to the fact that the beads shall not be able to escape from the column in which the expanded bed

25 has been created. Other factors which influence the choice of range limits for bead sizes and distribution within said range include the desired capacity and the substance or substances to be separated from the sample. Although less preferred, another alternative is to vary the density of the

30 beads used in one and the same bed. Bead fractions in which both the size and the density of the individual beads vary can also be used.

The density (mean density) of the beads will always be > 1 g/cm^3, for instance ≥ 1.1 g/cm^3, such as ≥ 1.2 g/cm^3, and

35 upwards (measured in the buffer used to maintain the bed in a fluidized state). Beads that are used in one and the same bed will preferably have generally the same density. The amount of filler required can be readily determined from a given polymer base matrix and from the density desired.

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It is preferred that the beads are porous with open pores. Optimal porosity can be determined on the basis of the substance or substances to be adsorbed among other things, and can be calculated conventionally. In order to achieve
5   effective adsorption and/or separation, Kav should lie in the range of 0.40-0.95 for the substance or substances to be adsorbed. For a definition of Kav see L. Hagel in "Protein Purification, Principles, High Resolution, and Applications", J-C Janson and L Rydén (Eds), VCH Publishers Inc. New York,
10 1989, p. 99.

The beads are normally comprised of a polymer base matrix in which the filler is enclosed. The polymer in the base matrix may be hydrophobic, for instance a bead which is comprised of styrene-divinyl benzene copolymer and which is
15 hydrophilized on the surface by coating it with an appropriate hydrophilic polymer (preferably a polymer having hydroxy or amino groups), for instance. Alternatively, the base matrix may be comprised of an insoluble or soluble hydrophilic polymer, for instance agarose, cellulose, dextran starch,
20 etc., which has been cross-linked to the degree of porosity and stability desired in a known manner, when necessary. At the time of filing the instant application, agarose was the polymer preferred, preferably in a cross-linked form.

The beads often have some form of affinity to the substance
25 or substances to be separated out. This normally means that the base matrix is substituted with one or more groups that have affinity to the substance/substances concerned. Typical groups are:

1. Positively charged groups (primary, secondary, tertiary
30   or quartenary amine groups).

2. Negatively charged groups (for instance carboxy, phosphonic acid, sulfonic acid, etc.).

3. Amphoteric groups.

4. Groups having a specific affinity (for instance bio-
35   affinity groups), such as between IgG-binding protein (Protein A, G, L, etc.) and IgG, lectin and carbohydrates, antigen/hapten and antibody, (strep) avidin and biotin.

5. Complementary nucleic acids/oligonucleotide).

6. Groups which exhibit pi-electron systems.

7. Chelate groups.

8. Hydrophobic groups, etc.

5   The matrix itself may also have an affinity to the substance or substances to be adsorbed, this affinity being expressed by virtue of the substance concerned being delayed when it shall pass a bed of non-substituted beads. With the aid of these groups, the inventive method can be performed as

10 affinity chromatography, such as ion-exchange chromatography, biospecific affinity chromatography, hydrophobic chromatography, "Reverse Phase Chromatography", chelate chromatography, covalent chromatography, etc. At the filing date of this application, the use of beads according to the above

15 substituted with IgG-binding protein, such as Protein A, G, H or L, preferably produced by recombinant techniques and optionally containing cysteine (for instance rProtein A-cys) was particularly preferred to affinity-purify IgG, particularly monoclonal antibodies. Alternatively, adsorption

20 can be effected batch-wise in a vessel in which the particles are fluidized by subjecting them to a flow of liquid or by agitation.

    The samples to be purified may be of the same type as those earlier used in chromatographic processes on packed or

25 expanded beds, or in batch adsorption processes on fluidized beds. The invention can be applied to great benefit in the direct treatmen of supernatants/culture media from fermentators and other cell culture vessels, particularly in chromatographic processes on expanded beds.

30   The invention will function for the separation of compounds of various molecular weights and types. Examples are polysaccharides, proteins/polypeptides and nucleic acids and synthetic water-soluble polymers, e.g. with molecular weights $\geq$ 5,000 dalton. Typically preferred molecular weights with

35 regard to substances to be adsorbed on the beads in accordance with the invention are > 50,000, preferably > 100,000. There is normally no upper limit, even though the process is normally limited to the removal of compounds that have a molecular weight below 350,000.

10

Manufacture of the beads, cross-linking, introduction of binding groups, addition of filler, etc., are effected in a known manner, while ensuring that the beads will be suited for adsorption purposes in accordance with the aforegoing.
5 The beads are sieved when necessary, to obtain a suitable size fraction.

The affinity group concerned may be introduced on a bead by activating an appropriate hydrophilic group, such as carboxy, amino, hydroxy, etc., with a suitable bifunctional reagent,
10 such as CNBr, bisepoxide or corresponding epihalohydrin, etc., which in is turn reacted with a compound that exhibits the affinity group concerned.

A second aspect of the invention, is a bead population (bead fraction) according to the above, which is suitable for
15 use as a matrix in adsorption processes, particularly chromatographic processes, effected on expanded/fluidized beds in accordance with the aforegoing. This aspect of the invention also includes the population in the form of a stable expanded bed according to the above, placed in a
20 chromatographic column. The aspect also includes bead populations which lack affinity groups, including preactivated forms. In this latter case, the customer/user can himself introduce a desired group.

A particularly important embodiment of this second aspect
25 of the invention includes beads which are substituted with IgG-binding protein, particularly an IgG-binding protein containing the amino acid cystein for mediating binding to the beads. Proteins of this type have earlier been produced by recombinant techniques, e.g. cysteine-variants of Protein
30 A (Ljungquist, et al, (Eur. J. Biochem. 186 (1989) 557-561) and Profy (EP 284,368), and cysteine-variants of Protein G (Fahnestock, et al (U.S. 4,977,247)). It is known to bind cysteine-containing proteins covalently to solid phases via thiol groups. It is also known that certain coupling methods
35 will give high selectivity for bonding via thiol, for instance via reactive disulphides (for instance pyridyl disulfide) or maleimide groups. Other methods, such as via epoxy or epihalo hydrin, are less selective and also give coupling via primary amine (lysyl).

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With the aid of IgG-binding protein, particularly recombinant Protein A-cys, we have been successful in producing filler matrices whose total capacity lies in the range above 30 mg IgG/ml gel, for instance between 40-60 mg
5   IgG/ml gel, and whose breakthrough capacity has been above 20 mg IgG/ml gel, for instance in the range of 20-30 mg IgG/ml gel (with $c/c_0 = 0.01$, where c is the IgG concentration in eluate and c_0 is IgG concentration in applied fluid). The base matrix comprised cross-linked agarose. In these
10 instances, the filler had the aforesaid particle form. See also the experimental part of this document.

The following experimental part discloses the manufacture of the bead population most preferred at the priority filing of this application. When derivatized further or used in
15 different studies, this bead population is referred to as standard gel.

EXPERIMENTAL PART

20 **SYNTHESIS METHODS**
   Fillers used:
   o   Anval 600® (Anval, Torshälla, Sweden) is a chromium-
       nickel alloy having the chemical composition (weight-%) C
       0.02%, Si 0.41%, Mn 0.31%, P 0.007%, S 0.001%, Cr 15.5%,
25   Ni 75.0%, Cu 0.02% and Fe 8.68%. The alloy has a density
       of 8.4 g/cm³. The particles have a spherical shape and
       preparations with two diameters were used (16-44 μm & <
       16 μm).
   o   Tantalum (Ta) (Novakemi AB, Enskede, Sweden), having a
30   density of 16.5 g/cm³ and consisting of sintered spheri-
       cal particles. Diameter 5-44 μm.
   o   Tungsten carbide (WC) (AB Sandvik Coromant, Stockholm,
       Sweden) having a density of 15.6 g/cm³ and consisting of
       small (in relation to tantalum) sintered spherical
35   particles. Two preparations were used (diameter 10-50 μm
       and < 15 μm).
   o   Zirconium oxide (ZrO₂) (MEL Chemicals, Manchester,
       England) having a density of 5.6 g/cm³ and consisting of
       small (in relation to tantalum) sintered spherical

particles. There was used a preparation having a diameter of 5-30 μm.

1.   **Emulsion**

5   **Step 1.1.   Dissolution of ethyl cellulose in the emulsifying reactor.**

Equipment: Cylindrical glass reactor 2.5 1 with flat bottom (emulsifying vessel), water bath, armature agitator and thermometer.

10   Method: 1050 ml of toluene here charged to the reactor, whereafter the agitator was activated and a 49.5 g of ethyl cellulose were added in a "fine jet". The mixture was heated to 60°C until all ethyl cellulose had dissolved (about two hours).

15

Step 1.2.   Preparing a solution containing agarose and filler.

Equipment: Mantled glass reactor with bottom valve (the reactor was positioned so that its contents could be
20   emptied into the emulsifying vessel), agitator, circulating water and thermometer.

Method: 900 ml of distilled water were charged to the reactor. The agitator was then activated and 36 g of agarose were delivered. The mixture was heated to 95°C
25   until the agarose had dissolved (about one hour). 378 g Anval® were then added to the mixture and the mixture was agitated for a further fifteen minutes, after which the temperature was lowered to 70°C.

30   **Step 1.3.   Transferring the solution containing agarose and filler to the emulsifying vessel.**

Method: The speed of the agitator in the emulsifying vessel was adjusted to 130 r.p.m. and the solution (70°C) was transferred to the emulsifying vessel.

35

Step 1.4.   Adjusting particle size.

This was effected conventionally, by varying the agitator speed.

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Target values: When desiring a gel in which the beads of the main bead fraction have a diameter of 80-160 μm, the emulsifying process is interrupted when 95% (volume) of the beads have a diameter < 200 μm (standard gel). When desiring bead diameters of 80-200 μm in the main bead fraction, the emulsifying process is interrupted when 95% of the beads have a diameter < 250 μm.

Step 1.5. Cooling.

Cooling: Heating of the water bath was stopped. With the equipment used (laboratory scale), the temperature of the bath was lowered from 60 to 30°C in about seven hours.

Step 1.6. Working up.

Method: The beads were washed by agitation and thereafter decantered (3x) with 3 l of 99.5% ethanol. Washing of the beads was continued on nutsch with 4 x 2 l of ethanol with self-draining. The beads were finally transferred to distilled water, via agitation and decantering.

2.   Cross-linking.

Equipment: Glass reactor, water bath, agitator, glass cooler and thermometer.

Method: 100 ml of 75% gel slurry and 34 g of sodium sulphate were charged to the reactor and stirred for two hours, whereafter the temperature was raised to 50°C. 1 ml of 45% NaOH solution and 0.1 g of sodium boron hydride were then added. 7 ml (10.5 g) of 45% NaOH solution and 7.5 ml of epichlorohydrin were added at the same time with the aid of a pump and over a period of six to eight hours. The reaction was allowed to continue overnight while stirring the system (for about sixteen hours) at 50 °C. Upon completion of the reaction, the gel was slurried (washed) with 7 x 150 ml water, whereafter the gel was acidified with 60% acetic acid to pH 5-6 and wet-sieved (80-160 μm or 80-200 μm).

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3.   Coupling Protein A to the finished gel.

Native Protein A was from Pharmacia Biotech AB, Sweden. Recombinant Protein A-cys (rProtein A-cys) contained the four IgG-binding domains (E, D, A, B and C) of the native form,

5   followed by the first eight amino acids of the X-domain, followed by a non-Protein A sequence of five amino acids and cysteine in the C-terminal (the Protein A sequence corresponded to alanine in position 18 up to and including proline in position 316 in accordance with EP 284,368. The

10 process was analogous with the process earlier described by Profy T (EP 284,268) and Lungquist, et al (Eur. J. Biochem. 186 (1989) 557-561). Solutions of rProtein A-cys were stored in a reduction buffer.

15 A.   Native Protein A

Coupling with N-hydrolxysuccinimide (NHS):   30 ml of standard gel were washed with distilled water, mixed with 3.67 g NaOH dissolved in 18 ml distilled water while stirring, and the temperature was adjusted to 24°C. After

20   some minutes, 7.2 ml of epichlorohydrin were added while vigorously stirring the mixture. After two hours, the gel was washed on a glass filter with 300 ml of distilled water. The washed gel was then mixed with 6-aminocapronic acid (6-ACS; 30 ml solution 1 M 6-ACS, 1 M NaCl pH 11.5)

25   and the mixture stirred for 17-24 hours and then finally washed with 200 ml 0.5 M NaCl. The gel was then again washed, now with 2 x 30 ml acetone, whereafter the gel was mixed with 15 ml acetone and activated with 559 mg NHS and 1007 mg of dicyclohexylcarbodiimide while

30   stirring the system. After 4-17 hours at 31°C, the gel was washed with 2 x 30 ml acetone + 450 ml isopropanol and cooled with 210 ml of ice-cold 1 mM HCl. The resultant activated gel was mixed with 30 ml solution containing native Protein A (Protein A dissolved in 0.2 M

35   NaHCO$_3$, 1 M NaCl pH 8), and stirred at room temperature for two hours, and then washed with Tris buffer pH 8, and acetate buffer pH 3, and finally with distilled water.

15

B.   Coupling of rProtein A-cys

Activation with the aid of 1,4-bis (2,3-epoxypropoxy) bu-
tane (BPR butane).   One litre of drained Sepharose® FF
(agarose in bead form cross-linked with epichlorohydrin,
Pharmacia Biotech AB, Uppsala, Sweden) was washed on
nutsch with distilled water and mixed in a thermostated
reaction vessel with 55 g NaOH dissolved in 300 ml
distilled water, 35°C, while stirring the system. 390 ml
BPR butane were added. The system was stirred for two
hours at 35°C, followed by washing with 15 l water.

Coupling of rProtein A-cys.   The activated gel was washed
on nutsch with 3 x 1 l nitrogen-gas saturated 0.1 M Na
phosphate, 1 mM EDTA, pH 8.5 and allowed to drain. The
gel was then mixed with 5.5 g rProtein A-cys dissolved in
nitrogen-gas saturated aqueous solution of 0.1 M Na
phosphate, 1 mM EDTA, pH 8.5. The system was stirred at
37°C while blowing-in nitrogen gas. Sodium sulphate (370
g) was added. After stirring the system for two hours at
37°C, the gel was washed with 3 l of distilled water and
sucked dry until cracks formed.

Deactivation: The dry-sucked gel was mixed with 100 ml
glycerol dissolved in 900 ml 0.2 M sodium bicarbonate,
0.5 M NaCl, 1 mM EDTA, pH 10, while stirring the system.
The system was stirred overnight at 37°C, whereafter the
gel was pH-washed on nutsch with 0.1 M Tris, 0.15 M NaCl,
pH 8, and 0.05 M acetic acid in three cycles with a 3 x 1
gel volume in each cycle. The gel was finally washed with
water.

The aforesaid synthesis conditions were varied as disclosed
in the following Tables.

Analysis methods

Function tests on finished gel.

Degree of expansion

Equipment: Pump P 6000 and column XK 26/60 (Pharmacia
Biotech AB, Uppsala, Sweden).

Method: About 70 ml of finished gel were delivered to the
column. An upper adapter (without net) was fitted, such that

the distance to the lower adapter was about 52 cm. Elutration was effected over a period of one hour at a rate of flow of about 100 cm/h higher than that at which it is desired to determine the degree of expansion. At the end of the elutra-
5 tion process, the rate of flow was lowered to the flow rate at which the degree of expansion shall be determined. The gel height (H) was read-off after a further thirty minutes and the pump shut-off. When the gel had settled to form a sediment, fifteen minutes, the gel height (H_0) was again
10 read-off. The degree of gel expansion is determined from the H/H_0 relationship.

Breakthrough capacity Q_b (binding of IgG) at 300 cm/h ($c/c_0=0.01$). Packed bed. Native Protein A and rProtein A-cys.
15 Equipment

Instruments: FPLC with two P 6000 pumps (Pharmacia Biotech AB), UV-2 and printer.
Column: XK 16/20 (Pharmacia Biotech AB).
Buffer A: 20 mM sodium phosphate, pH 7.0.
20 Buffer B: 0.1 M glycine, pH 3.0.
Protein: hIgG (Pharmacia Biotech AB).
/ Flow: 10 ml/min. --> 300 cm/h.

Procedure: 25 ml of drained-off Protein A gel (or rProtein A-cys gel produced from standard gel in accordance with the
25 above) .were allowed to settle and form a sediment in the column. The adapter was adjusted to the gel surface and a flow containing buffer A was applied stepwise to 10 ml/min, whereafter the adapter was further adjusted. A flow of 0.5 g IgG per ml in buffer A was then applied through the column.
30 The column was initially bypassed and the flow delivered directly to the UV-monitor, wherein an absorbance value (280 nm) for non-absorbed solution was measured (c_0), whereafter the flow together with sample was allowed to pass through the column. When the absorbance value for the flow through the
35 column (280 nm) was 1% of the absorbance c_0, the test was interrupted, the gel was washed and IgG bound to the gel was eluated with buffer B and the gel washed with 0.1 M glycine, pH 2.5, 30% isopropanol and 20% ethanol. The eluate was collected and its IgG content determined, which in turn gave

the amount of absorbed IgG per ml of gel (breakthrough capacity (Q_B) for $c/c_0=0.1$).

The breakthrough capacity Q_B (IgG) for an expanded bed.
Native Protein A.

Equipment:

Instrument: Pump P 6000, UV-2; 280 nm and printer (Pharmacia Biotech AB).

Column: XK 26/60 (Pharmacia Biotech AB).

Buffer A: 20 mM sodium phosphate, pH 7.0.

Buffer B: 0.1 M glycine, pH 3.0.

Protein: 0.5 mg/ml hIgG (Pharmacia Biotech AB) in buffer A.

Flow: 300 cm/h.

Procedure: 12.8 cm of Protein A gel sediment (produced from the standard gel in accordance with the above) was elutriated at 400 cm/h and expanded stepwise in XK 26/60 (Pharmacia Biotech AB). The column was initially bypassed and the flow delivered direct to the UV monitor, in which an absorbance value (280 nm) of non-absorbed solution was measured (c_0), whereafter the flow together with sample was allowed to pass through the column. When the absorbance of the flow through the column (280 nm) was 1% of the absorbance c_0, the test was interrupted, the gel was washed and the IgG bound to the gel eluated with buffer B and washed with 0.1 M glycine, pH 2.5, 30% isopropanol and 20% ethanol. The eluate was collected and its IgG content determined, which in turn gave the amount of absorbed IgG per ml of gel (the breakthrough capacity (Q_B) for $c/c_0=0.01$).

Total capacity (IgG). Packed bed. Native Protein A and rProtein A-cys.

Equipment:

Instrument: FPLC system with superloop (Pharmacia Biotech AB).

Column: 1 ml Hitrap (Pharmacia Biotech AB).

Buffer A: 10 mM sodium dihydrophosphate, 0.15 M sodium chloride and 10 mm EDTA, pH 7.0.

Buffer B: 0.5 M acetic acid (gives a pH of about 2.7).

IgG solution: 150 mg hIgG in 10 ml of buffer A.

<u>Procedure</u>:   1.0 ml of Protein A gel (or Protein A-cys gel produced from standard gel in accordance with the aforegoing) was packed in the column and equilibrated with buffer A, whereafter IgG solution was delivered through the superloop at a flow rate of 0.15 ml/min. After washing with buffer A at the same rate of flow, adsorbed IgG was eluated with 9 ml buffer B at a flow rate of 0.30 ml/min. The eluate was collected and its volume determined. A_{280} was read-off after diluting to 1:10 and the total IgG capacity (mg (IgG/ml gel) was determined as the eluate volume (ml) x A_{280} (diluted eluate) x 7.244.

Table 1. Presentation of synthesis conditions and results from examining the relationships between filler (type and diameter) and the IgG capacity of Protein A (native) gel.

Agarose sol.		Filler			Emulg.	Sieving	Capacity	
		Type	Diam	Amount			Q_B	Total
ml	%		μm	g[1]	g[1]	μm	mg	IgG/ml gel
400	4	None			7.5	80-160	14.80	24.0
300	4	Quartz	<25	17	6.7	80-160	3.00	20.50
400	4	W[2]	<15	40	10	80-160	5.40	23.40
400	.4	W[2]	10-50	40	7.5	80-160	10.60	22.50
400	4	Tantalum	5-45	40	10	80-160	14.60	26.70
300	4	Anval	<16	40	5.8	80-160	11.60	19.80
300	4	Anval	16-20	40	7.5	80-160	14.90	23.30
400	4	Anval	16-44	40	10	60-160	15.30	23.20
300	4	Anval	44-105	40	7.5	3)	3)	3)
300	4	Z[4]	<15	40	7.3	80-160	9	17.00

1) Amount per 100 ml agarose solution; 2) Tungsten carbide; 3) Low emulsifying yield and hence cross-linking and coupling to Protein A were not carried out; 4) Zirconium oxide.

Table 2. Presentation of representative synthesis conditions and results from tests concerning the relationships between

added amounts of Anval to agarose solution and the degree of expansion of the cross-linked gel at 500 cm/h and 600 cm/h.

Filler[1] amount g[2]	Bead fraction μm	Degree of expansion (H/H_0) Flow 500 cm/h	Flow 600 cm/h
60	80-200	3.3	3.7
60	125-250	3.3	3.6
70	125-250	2.0	2.2
80	80-250	3.0	3.2
80	125-250	2.1	2.5

[1]Anval diameter 16-44 μm; [2]Amounts in Table calculated on 100 ml of agarose solution (4%).

The density varies between 1.6-1.8.

Table 3. Total capacity and breakthrough capacity Q_B (mg hIgG/ml sedimented gel, $c/c_0 = 1\%$) for Protein A gel (produced from standard gel in accordance with the experimental part).

Type of bed	Flow rate	Q_B	Total capacity
Packed bed	300 cm/h	15.8 mg/ml	25.1 mg/ml
Expanded bed	300 cm/h	13.4 mg/ml	Not determined

Table 4. Packed bed. Total capacity and breakthrough capacity Q_B (mg hIgG/ml sedimented gel, $c/c_0 = 1\%$) for rProtein A-cys gel (produced from epoxy-activated standard gel according to the experimental part).

Standard gel	Epoxy μmol/ml gel	Protein A-cys Charged mg/ml gel	mg hIgG per ml gel Total	Q_B
80-200 μ	6.0	6.5	52.7	30.0
80-200 μ	6.1	6.5	48.2	27.1
100-200 μ	5.9	6.5	49.2	21.1
80-200 μ	7.7	6.5	51.4	26.6

Characterizing the stability of an expanded bed

A column (200 mm, 100 cm height) containing a distributor whose hole area was 0.3% of the total area, was filled with 15 cm (4.7 l rProtein A-cys gel) having a particle size
5 distribution in the range of 80-160 µm. The gel was expanded to 38 cm at a linear flow rate of 300 cm/h (20 mM Na phosphate, pH 7.0). The pressure drop across the distributor was 100 Pa. A positive step-response injection with 0.25% acetone solution was introduced into the column as a stimulus
10 experiment. When 100% of the acetone solution could be detected at the column outlet, the flow was switched back to buffer solution for a negative step response. The plate number for the column and system was calculated on the basis of the negative step response in accordance with the same
15 principle as that applied with pulse injection (Chemical Reaction Engineering, 2nd Edition, John Wiley & Sons (1971)). The number of plates for the column plus system was 40. Compensation was then made for the number of plates for the system contribution, resulting in 34 plates for the column.
20 In turn, this corresponded to a vessel dispersion number of 17×10^{-3}.

DISCUSSION

Degree of expansion

25 The test results show that the inventive filler matrices are able to achieve degrees of expansion that lie within the ranges used in practice in expanded beds, even in the case of elevated flow rates (> 300 cm/h).

30 **Filler**

Synthesis conditions and results are apparent from Table 1. The fillers used were Anval®, tantalum, tungsten carbide, zirconium oxide and quartz.

There seems to be no differences for various fillers in the
35 total capacity of the gel to bind IgG.

The breakthrough capacity is significantly influenced by the type of filler used and its diameter and shape. Studied fillers can be divided into groups:

  o   Group 1 - Approximate breakthrough capacity (IgG) for gel with and without filler.

     Anval®:   Diameter 16-20 μm spherical particles.

     Anval®:   Diameter 16-44 μm spherical particles.

     Tantalum: Diameter 5-44 μm sintered spherical particles.

  o   Group 2 - About 25% lower breakthrough capacity (IgG) than Group 1.

     Anval®:   Diameter < 16 μm spherical particles.

     Tungsten carbide: Diameter 10-50 μm sintered small[1] spherical particles.

     Zirconium oxide: Diameter 5-30 μm sintered small[1] spherical particles.

  o   Group 3 - Roughly a 70% lower breakthrough capacity (IgG) than Group 1.

     Tungsten carbide: Diameter < 15 μm sintered small[1] spherical particles.

     Quartz: Diameter < 25 μm "crush-like particles".

[1]In relation to tantalum.

Comparison between fillers of equivalent diameters.

  o   Anval® (16-44 μm) and tantalum (5-44 μm) give the same breakthrough capacity (IgG) as gel that lacks a filler.

  o   Tungsten carbide (10-50 μm) gives a breakthrough capacity (IgG) which is about 30% lower than Anval® (16-44 μm).

  o   Tungsten carbide (< 15 μm) gives a breakthrough capacity (IgG) which is about 50% lower than Anval® (< 16 μm).

  o   Quartz (< 25 μm) gives a breakthrough capacity (IgG) which is about 75% lower than Anval® (< 16 μm) and about 40% lower than tungsten carbide (< 15 μm).

The usefulness of the various fillers with respect to breakthrough capacity (IgG) can be ranked on this basis: Anval® = tantalum > tungsten carbide > quartz. If it is assumed that the breakthrough capacity is influenced solely by shape, a smooth, spherical surface (Anval®) is better than

the surface of small, spherical sintered particles (tungsten carbide). Worst of all are the irregular particles ("crush" (quartz)). It can be seen from the study that quartz included in existing matrices for Streamline® is least suited as a
5   filler.

When comparing the same type of fillers with different diameters, it is found that:

o   Anval® - Having a particle diameter < 16 μm gives a breakthrough capacity (IgG) which is about 25% lower than
10   particles having a diameter of 16-44 μm.

o   Tungsten carbide - Having a particle diameter < 15 μm gives a breakthrough capacity (IgG) which is about 50% lower than particles sizes of 10-50 μm.

Filler having a particle diameter < 15 μm thus lowers the
15 breakthrough capacity (IgG).

Very low yields were achieved when emulsifying together with Anval® having a particle size distribution in the interval of 45-105 μm, because the filler sedimented instead of being incorporated in the beads.

20 The study carried out on the matrices shows that the optimal diameter of the filler particles is 15-40 μm with respect to manufacturing yield.

In order to achieve high mass transfer and thereby high breakthrough capacity at high flow rate, a preferred mode
25 employs, bead diameters < 80 μm, when not taking the entire system solution into account. There is often used at the inlet and outlet of chromatographic expanded beds a net which functions to retain the bed while allowing fermentor solution to pass through (including particulate impurities). In the
30 case of existing Streamline® , this means that particulate impurities of up to 56 μm in diameter are able to pass through. The upper bead diameter should preferably lie as close as possible to the lower bead diameter, if optimal capacity is to be obtained. In this study, we have obtained
35 good yields and high breakthrough capacity with the upper limit of 160-200 μm. The end choice of an upper limit will depend on the particle size distribution obtained by scaling-up relative the costs for the to base matrix and performance.

Stability of expanded beds comprising inventive beads.

The test results showed that expanded beds which fulfil conditions for stabilized beds can be obtained with good margins.

CLAIMS

1. A method for adsorbing in a fluidized bed containing beads in the form of a filler matrix to separate out from a sample solution one or more substances that has/have affinity to the beads, **characterized** by using as the matrix a bead population according to Claims 4-13.

2. A method according to Claim 1, **characterized** in that the fluidized bed is a stable expanded bed, preferably having a vessel dispersion number which is $\leq 75 \times 10^{-3}$.

3. A method according to Claim 1 or Claim 2, **characterized** by using beads substituted with groups that exhibit affinity to the substance or substances to be separated out.

4. A population of beads having a density > 1 g/cm^{-3} and constructed of a polymer base matrix in which filler particles are incorporated, **characterized** in that the filler particles have a density of ≥ 3 g/cm^{-3}.

5. A population of beads according to Claim 4, **characterized** in that the individual beads have different densities within a given density range and/or different sizes within a given size range.

6. A population of beads according to Claim 4 or Claim 5, **characterized** in that the beads have a mean density of ≥ 1.2 g/cm^{-3}.

7. A population of beads according to any one of Claims 4-6, **characterized** in that the beads have a mean size of between 10-1,000 μm, preferably between 50-700 μm.

8. A population of beads according to any one of Claims 4-7, **characterized** in that the filler has a particle size in the range of 1-70 μm, preferably between 15-50 μm.

9.   A population of beads according to any one of Claims 4-8, **characterized** in that the filler particles have a rounded shape or an aggregate/agglomerate of particles of such shapes.

5

10. A population of beads according to any one of Claims 4-9, **characterized** in that the filler particles have a spherical or ellipsoidal shape.

10 11. A population of beads according to any one of Claims 4-10, **characterized** in that the filler particles contain a heavy metal, preferably in the form of a heavy metal salt, such as a carbide or an oxide, or an alloy or a pure metal.

15 12. A population of beads according to any one of Claims 4-11, **characterized** in that the polymer base matrix includes a plurality of hydroxy groups.

13. A population of beads according to any one of Claims 4-
20 12, **characterized** in that the beads are porous.

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